

WHAT IS CLAIMED IS:

1. A method for performing multiplexed determinations of target nucleic acid where modified primer reagents are captured and selectively released, each primer reagent
5 comprising a first sequence for hybridizing to a target and a second sequence for capture, a capture reagent comprising a homologous sequence to each of said second sequences, and a displacement reagent comprising a nucleic acid sequence homologous to each of said capture sequences, and wherein a modifying reagent system is employed for modifying primer reagent bound to target nucleic acid, said method comprising:

10 combining target nucleic acid with a plurality of said primer reagent comprising differing second sequences for differing first sequences, under hybridizing conditions in the presence of said modifying reagent system in an assay mixture, whereby primer reagent bound to target nucleic acid is modified;

15 combining modified primer reagent with said capture reagent at a single site, whereby modified primer reagent is separated from other components in said assay mixture; releasing sequentially said captured, modified primer reagent with said displacement reagent; and determining said modified primer reagent.

20 2. A method according to Claim 1, wherein said modifying reagent system is a sequencing reaction system.

25 3. A method according to Claim 1, wherein said multiplexed determination is a multiplexed genotype determination of single base positions in said target nucleic acid.

4. A method for performing multiplexed determinations of target nucleic acid using a combination of reagents, comprising: (1) a primer reagent comprising a mixture of primer oligonucleotides each having (a) a unique primer first part sequence homologous to a target nucleic acid sequence, (b) a primer second part sequence having a sequence homologous to a

capture sequence, said primer reagent comprising a plurality of second part sequences, with the proviso that where each of said second part sequences are unique within the group of primer oligonucleotides, optionally said first part serves as the primer first and second part sequences; (2) a capture reagent for sequestering said primer oligonucleotides, comprising a plurality of capture oligonucleotides each having a capture sequence homologous to primer second part sequences; and (3) strand displacement reagents, each comprising a strand displacement oligonucleotide having a sequence homologous to a different said capture oligonucleotide for displacement of said primer oligonucleotides; said method comprising:

- (a) combining under hybridizing conditions said target nucleic acid and said primer reagent, whereby said primer oligonucleotides hybridize to homologous sequences present in said target to form primer duplexes;
- (b) enzymatically modifying said primer oligonucleotides in said primer duplexes to change by at least one nucleotide said primer oligonucleotide to produce modified primer oligonucleotides;
- (c) sequestering said modified primer oligonucleotides with said capture oligonucleotides;
- (d) adding a strand displacement reagent under strand displacement conditions, whereby primer oligonucleotides are released from the sequestering moiety to provide a separated portion containing a mixture of modified primers having common capture sequences, which are also common to said added strand displacement reagent; and
- (e) assaying said separated portion of modified primers to provide said multiplexed determination.

5. A method according to Claim 4, wherein steps (d) and (e) are repeated a plurality of times, using in each step (d) different said strand displacement reagents.

6. A method according to Claim 5, wherein following step (b), said method comprises the additional steps of:

denaturing said primer duplexes; and

repeating steps (a), (b) and said denaturing to produce additional modified primer

sequences.

7. A method for performing multiplexed determinations of target DNA using a combination of reagents, comprising: (1) a primer reagent comprising a mixture of primer oligonucleotides each having (a) a unique primer first part sequence homologous to a target nucleic acid sequence, (b) a primer second part sequence having a sequence homologous to a capture sequence, said primer reagent comprising a plurality of second part sequences, with the proviso that where each of said second part sequences are unique within the group of primer oligonucleotides, optionally said first part serves as the primer first and second part sequences; (2) a capture reagent for sequestering said primer oligonucleotides, comprising a plurality of capture oligonucleotides having (a) capture first part sequences homologous to primer second part sequences, and (b) capture second part sequences homologous to a portion of a strand displacement oligonucleotide; and (3) strand displacement reagents, each comprising a strand displacement oligonucleotide having a sequence homologous to a different said capture oligonucleotide for displacement of said primer oligonucleotides; said method comprising:

- (a) combining under hybridizing conditions said target DNA and said primer reagent, whereby said primer oligonucleotides hybridize to homologous sequences present in said target to form primer duplexes;
- (c) enzymatically modifying said primer oligonucleotides in said primer duplexes to change by at least one nucleotide said primer oligonucleotide to produce modified primer oligonucleotides;
- (c) sequestering said modified primer oligonucleotides with said capture oligonucleotides;
- (d) adding a strand displacement reagent under strand displacement conditions, whereby primer oligonucleotides are released from the sequestering moiety to provide a separated portion containing a mixture of modified primers having common capture sequences;
- (e) repeating step (d) until all of said strand displacement reagents have been added; and
- (f) assaying said separated portions of modified primers to provide said multiplexed determination.

9. A method according to Claim 7, wherein said capture oligonucleotides are combined with said modified primer oligonucleotides to form primer/capture duplexes prior to linking said capture oligonucleotides with a support.

11. A method according to ~~Claim~~ 7, wherein said primer oligonucleotides comprise an identifier moiety specific to each of said primer oligonucleotides.

13. A method according to Claim 12, wherein said extending is performed in four different vessels, each vessel having a different terminating nucleotide and at least one of said primer reagent and said terminating nucleotide is labeled to identify the terminating nucleotide of said extended primer.

14. A method for performing multiplexed determinations of target DNA to determine a multiplicity of greater than about 50 genotypes at single positions, using a combination of reagents, comprising: (1) a primer reagent comprising a mixture of primer oligonucleotides each having (a) a unique primer first part sequence homologous to a target nucleic acid sequence wherein the 3' terminal nucleotide is directly adjacent to a single base position of interest; (b) a primer second part sequence homologous to a capture sequence; and (c) an identifier moiety identifying said primer oligonucleotide, wherein said primer oligonucleotides have a plurality of different primer second part sequences; (2) a capture reagent for sequestering said primer oligonucleotides, comprising a plurality of capture oligonucleotides having (a) capture first part sequences homologous to the primer second part sequences, and

(b) capture second part sequences homologous to a portion of a strand displacement oligonucleotide; and (3) strand displacement reagents, each comprising a strand displacement oligonucleotide having a sequence homologous to a different said capture oligonucleotide for displacement of said primer oligonucleotides; said method comprising:

- 5 (a) combining under hybridizing conditions said target DNA and said primer reagent, whereby said primer oligonucleotides hybridize to homologous sequences present in said target DNA to form primer duplexes;
- (b) extending said primer oligonucleotides in said primer duplexes with a polymerase to add one terminating nucleotide to said primer oligonucleotide to form extended primer sequences;
- 10 (c) dissociating said extended primer sequences from homologous sequences;
- (d) repeating steps (a), (b) and (c) to produce additional extended primer sequences;
- (e) sequestering said extended primer sequences with said capture oligonucleotides;
- (f) adding a strand displacement reagent under strand displacement conditions, whereby
- 15 primer oligonucleotides are released from the sequestering moiety to provide a separated portion of a mixture of extended primers having common capture sequences;
- (g) repeating step (f) until all of said strand displacement reagents have been added; and
- (h) assaying said separated portions of extended primers to provide said genotype determination.

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15. A method according to Claim 14, wherein said extending is performed in four different vessels, each vessel having a different terminating nucleotide and at least one of said primer reagent and said terminating nucleotide is labeled to identify said terminating nucleotide.

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16. A method according to Claim 14, wherein said extending is performed in a single vessel with four different terminating nucleotides, each terminating nucleotide labeled with a different label to identify said terminating nucleotide.

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17. A method according to ~~Claim 14~~, wherein said identifier is a mobility tag for electrophoretic separations.

19. A method for performing multiplexed sequencing of target DNA comprising at least about 5 kb fragments using a combination of reagents, comprising: (1) a primer reagent comprising a mixture of primer oligonucleotides each having (a) a unique primer first part sequence homologous to a target nucleic acid sequence and (b) a primer second part sequence homologous to a capture sequence, said primer oligonucleotides comprising a plurality of second part sequences, with the proviso that optionally said first part serves as the primer first and second parts; (2) a capture reagent for sequestering said primer oligonucleotides, comprising a plurality of capture oligonucleotides having (a) capture first part sequences homologous to the primer second part sequences, and (b) capture second part sequences homologous to a portion of a strand displacement oligonucleotide; and (3) strand displacement reagents, each comprising a strand displacement oligonucleotide having a sequence homologous to a different said capture oligonucleotide for displacement of said primer oligonucleotides; and (4) a template-dependent extension terminator; said method comprising:

- (a) combining under hybridizing conditions said target DNA and said primer reagent, whereby said primer oligonucleotides hybridize to homologous sequences present in said target DNA to form primer duplexes;
- (b) extending said primer oligonucleotides in said primer duplexes with a polymerase in the presence of dNTPs and at least one terminator nucleotide, to add said dNTPs and said at least one terminator nucleotide to said primer oligonucleotide to extend said primer oligonucleotide with a sequence complementary to the DNA target sequence to form extended primer sequences, with the proviso that the extending will include the four different terminator nucleotides in the same or different vessels;
- (c) dissociating said extended primer sequences from homologous sequences;
- (d) repeating steps (a), (b) and (c) to produce additional extended primer sequences;
- (e) sequestering said extended primer sequences with said capture oligonucleotides;

(f) adding sequentially said strand displacement reagents under strand displacement conditions, whereby primer oligonucleotides are released from the sequestering moiety to provide separated portions of mixtures of extended primers of different lengths having common capture sequences; and

5 (g) assaying said separated portions of extended primers to determine the sequence of said target DNA.

20. A method according to Claim 19, wherein said target DNA derives from one contiguous DNA strand.

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21. A method according to Claim 19 wherein said target DNA derives from at least two separate DNA strands.

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22. A method according to Claim 19, wherein said extending is performed in four different vessels, each vessel having a different terminating nucleotide and one of said primer reagent or said terminating nucleotide is labeled to identify said terminating nucleotide.

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23. A method according to Claim 19, wherein said extending is performed in a single vessel with four different terminating nucleotides, each terminating nucleotide labeled with a different label to identify said terminating nucleotide.

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24. A method for multiplexed sequencing of target DNA using a combination of reagents comprising (1) a primer reagent comprising a plurality of primer oligonucleotides each having (a) a unique primer first part sequence homologous to a target nucleic acid sequence, which also serves as a capture sequence; (2) a capture reagent for sequestering said primer oligonucleotides, comprising a plurality of capture oligonucleotides having (a) capture first part sequences homologous to the primer first part sequences, and (b) capture second part sequences homologous to a portion of a strand displacement oligonucleotide and (c) linked to a sequestering moiety; (3) strand displacement reagents, each comprising a strand displacement oligonucleotide having a sequence homologous to a different said capture

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oligonucleotide for displacement of said primer oligonucleotides; and (4) a template-dependent extension terminator; with the proviso that one of said primer oligonucleotides or said extension terminator is labeled with a detectable label, said method comprising:

- (a) combining said target DNA, said primer reagent and said template dependent extension terminator under conditions for hybridization and chain extension, whereby said primer oligonucleotides hybridize to and are extended along said target DNA to form extended primers hybridized to said target DNA;
- (b) dissociating said extended primers from said target DNA;
- (c) repeating said combining and dissociating steps to provide sufficient numbers of extended primers for sequencing of said target DNA;
- (d) sequestering said extended primer sequences with said capture oligonucleotides;
- (e) adding sequentially said strand displacement reagents under strand displacement conditions, whereby the extended primers corresponding to said strand displacement oligonucleotides are released in separate portions from said sequestering moiety; and
- (f) assaying said separated portions of extended primers to provide said multiplexed sequencing.

25. A kit comprising segregated components, said components comprising sets of at least one each of a primer reagent and a capture reagent, and strand displacement reagents, wherein:

- (1) said primer reagent comprises a mixture of primer oligonucleotides each having (a) a unique primer first part sequence homologous to a target nucleic acid sequence, (b) a primer second part sequence having a sequence homologous to a capture sequence, said primer reagent comprising a plurality of second part sequences, with the proviso that where said second part sequences are unique within the group of primer oligonucleotides, optionally said first part serves as the primer first and second part sequences; (2) said capture reagent serves for sequestering said primer oligonucleotides, comprising a plurality of capture oligonucleotides each having capture sequences homologous to said primer second part sequences; and (3) said strand displacement reagents comprise a strand displacement oligonucleotide each having a sequence homologous to a different said capture oligonucleotide for displacement of said primer oligonucleotides.

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29. A kit according to Claim 25, wherein said primer oligonucleotides comprise an identifier moiety specific to each primer oligonucleotide.